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Studies on the Biosynthesis of Pyocyanine. (V)

On the Bacterial Mutation from the Viewpoint of Pigmentation

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An erratic pigmentation of the bacteria was found to be based on the bacterial mutation which occurred spontaneously under unknown circumstances. The experiment with a mutant strain has succeeded in the formation of pyocyanine by the technique that the accumulation product of the normal strain cultured in the presence of inhibitory agent, or the culture extract of the other mutant strain was administered to the strain to be incubated. According to these facts some discussions were brought up concerning the bacterial mutation from a standpoint of the mechanism of pyocyanine formation.

INTRODUCTION

As was already mentioned, the formation of pyocyanine is easily affected by various cultural conditions such as the composition or the pH of the medium and the cultural temperature[1].

However, there has sometimes been observed the phenomenon that even under the same condition, pyocyanine formation is remarkably decreased whereas the bacterial growth is satisfactory. This fact was found to be due to the bacterial mutation : the normal strain incubated might be contaminated by the other pigmentless strain which had been derived from the former as a mutant strain.

On the other hand, such a strain has been recognized which revealed pyocyanine formation at the same level as was observed in peptone medium, even in the synthetic medium containing urea as a sole source of nitrogen. In view of these facts, it has become difficult to define strictly the cultural condition for pyocyanine formation, unless the strain supplied to the experiment was limited to one strain.

On the contrary, however, a clue may be offered for the study of the mechanism of pyocyanine biosynthesis, by using the mutant strain for the experiment. And it has become possible to expect that when no pigmentation was performed according to the cultural condition, an intermediate in pyocyanine synthesis could be accumulated in the culture medium, because the experiment has succeeded in the formation of pyocyanine by the mutant strain which has lost the ability to form pyocyanine, by administering the fraction of the cultured solution of the normal strain in which no pigment was produced. In general, if the intermediate might be accumulated by the addition of the inhibitory agent of pigmentation, the accumulation product would be expected to vary in its kind, in response to the variety of the inhibitors. After the manner similer to this

mechanism, it has been expected that the accumulation product in the medium of the mutant strain was divided according to the kind of the strain.

It is of interest to note that even with the mutant strain which occurs spontaneously under the same circumstance, the types of the mutation may not necessarily be the same with one another. As for the mutation, besides the changes in pigmentation, such variations as in assimilation or production of some organic acids, in resistance to certain antibiotic substance and in the form of colony were observed. In the present work, the bacterial mutation will be discussed mainly from the viewpoint of pigmentation so as to concern the mechanism of biosynthesis of pyocyanine.

EXPERIMENTAL AND DISCUSSION

Inhibition of Pyocyanine Formation

As was previously reported, at a high level of phosphate pyocyanine forma-

Table 1. Inhibitory action on pyocyanine formation and bacterial growth.

Inhibitors (%)	0.4	0.2	0.1	0.05	0.025	0.010	0.005	0.0025
K_2HPO_4	(−) ###	(−) ###	(−) ###	(+) ###	(###) ###	(###) ###	(###) ###	(##) ##
Streptomycin	(−) ##	(−) ##	(−) ##	(+) ###	(##) ###	(##) ###	(##) ###	(###) ###
Penicillin	(−) +	(##) ##	(##) ##	(###) ###	(###) ###	(###) ###	(###) ###	(###) ###
H_2O_2	×	×	×	(##) ##	(###) ###	(###) ###	(###) ###	(###) ###
KIO_4	×	×	×	×	(−) ##	(−) ##	(##) ##	(##) ###
Formaldehyde	×	×	×	×	×	×	(##) ##	(##) ###
Arsenic acid	×	×	(−) +	(−) ##	(−) ##	(−) ##	(−) ###	(−) ###
$AgNO_3$	×	×	×	×	×	×	(−) +	(trace) ##
$HgCl_2$	×	×	×	×	×	×	×	(−) +
$CoCl_26H_2O$	×	×	(−) ##	(−) ##	(trace) ##	(##) ##	(##) ###	(##) ###
$CuSO_45H_2O$	×	(−) ##	(−) ##	(−) ##	(trace) ##	(##) ##	(##) ###	(###) ###
Phenol	×	×	(−) ##	(−) ##	(trace) ##	(+) ###	(##) ##	(##) ###
Resorcinol	×	×	(−) +	(−) ##	(−) ##	(+) ##	(+) ###	(##) ###

Basal medium : 2.5% glycerol, 1% petone, 0.2% urea, 0.025% $MgSO_47H_2O$ and 0.025% K_2HPO_4 are contained, pH 7.4.

Strain B_1 was incubated at 37° for 4 days. (−), (+), (##), (###), (###) Represent respective amounts of pyocyanine of zero, 0.002, 0.005, 0.01 and 0.02%. ###, ##, ##, + ; Bacterial cell numbers of 10^9, 5×10^8, 2×10^8 and 10^8 in rough estimation ; ×, No multiplication.

tion cannot be revealed at all, whereas the bacterial growth is satisfactory. Similar inhibitory action was often observed with the cases of some heavy metals, antibiotics and other toxic substances. In most cases, a discrepancy of the limiting concentrations is usually observed : pyocyanine formation is remarkably inhibited at lower concentration of inhibitory agents, whereas the bacterial growth is considerably satisfactory at higher concentration. And it is suggested that the enzyme system in pyocyanine synthesis is far sensitively affected by these inhibitors than in baterial growth. In these cases, it would be expected that an intermediate product in pyocyanine formation might be accumulated in the medium, if the experiment with the pigmentless strain could be successful in revealing pyocyanine formation by the administration of the accumulation product.

In some cases, it was observed that both pigmentation and bacterial growth were simultaneously inhibited at the same concentration of the inhibitory agents such as Ag^+, Hg^+, hydrogen peroxide and formaldehyde (Table 1).

It is familiar with various kinds of micro-organisms that resistant ability of the organism to antibiotics such as penicillin or streptomycin, or to virus can be induced by the antigenic agent itself[2~6]. It was of considerable interest that the induction of bacterial resistance to streptomycin could be observed not only on bacterial growth but also on pyocyanine formation.

Similar phenomenon was recognized with arsenite, periodate or copper ion, although these substances did not produce so remarkable effects as streptomycin.

Occurrence of Spontaneous Mutation

It is an well known fact that change in morphological, serological or biochemical nature of bacteria occurs suddenly or unpredicably. In the present study, the author has frequently encountered with the accidental difficulties due to the erratic pigmentation of the bacteria. The reason why the pigmentation was thus capricious, was found to be mostly ascribable to the spontaneous mutation of the bacteria which make, therefore, the unification of the condition for pyocyanine formation troublesome.

The parent strain to be termed the "prototrophic" exhibited the formation of pyocyanine only in peptone medium as had previously been presented[1]. Afterwards, the new strain has derived which could reveal appreciable pyocyanine formation in the synthetic medium without peptone—1 g of peptone was replaced by 0.5 g of glutamic acid—and subsequently in the medium containing urea as a sole source of nitrogen. In general, the formaton of pyocyanine in synthetic medium is of far small amount as compared with that in peptone medium. However, even in such simple synthetic medium, a remarkable pigmentation was revealed by the strain derived from the one mentioned above, as in the peptone medium or in the methionine-containing medium which has peecedingly been reported[7]. To the contrary, as mentioned before, such a strain has appeared which could never reveal any pigmentation whatever a suitable cultural contition might be provided. Thus, the conditions for pigmentation are diverse according to the kind of the bacterial strain. In Fig. 1, the variant strains regarded to be of genetic mutation are summarized, which have hitherto been

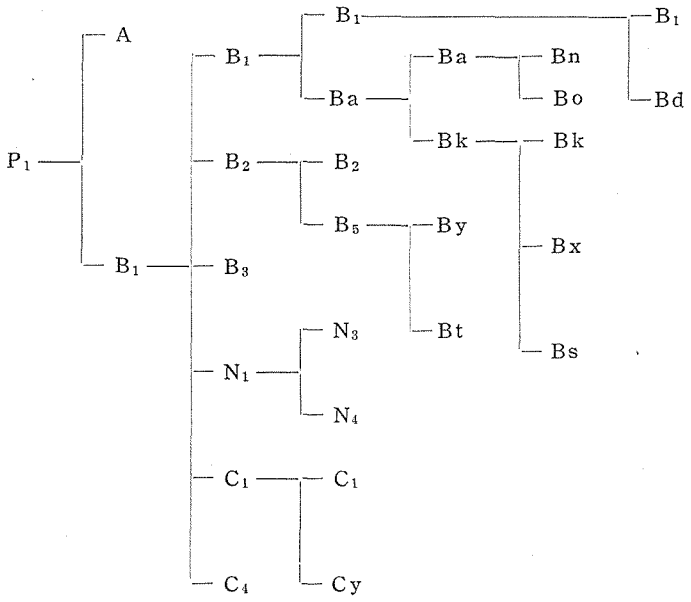

Fig. 1. Occurrence of spontaneous mutation.

isolated in this laboratory. In connection with the study on the bacterial mutation, some discussions will be brought up from the viewpoint on the mechanism of pyocyanine formation. The strain revealing the pigmentation in the synthetic medium containing glycerol and urea as sources of carbon and nitrogen should be regarded to possess the ability to prepare perfectly the factors necessary for pyocyanine formation. In general, peptone shows a promoting effect on pigmentation with major strains, in which the effective constituents have precedingly been ascertained to be methionine and other stimulating factors, so that major strains were expected to reveal an increasing pigmentation in the addition of methionine or of the stimulating factors in peptone. The fact that in spite of the remarkable pigmentation in peptone medium pyocyanine is hardly produced in synthetic medium, may be ascribed to the smaller capacity for the synthesis of methionine which may tolerably be synthesized by the bacteria with other amino acids to such a degree as its own growth is sustained, and/or simultaneously attributed to the inferior ability to synthesize the factor recognizable in peptone. Similarly, a poor pigmentation of the strain unresponsive to methionine can probably be based on the less bacterial capacity for the synthesis of the factor in peptone or of the other unknown factor.

In the present experiment, what is termed "mutant" will mean such a strian as hardly reveals or cannot at all the formation of pyocyanine in the medium which satisfies every condition known with the parent strain, although the major strains other than the parent type may belong to this category in a strict sence of the term. For the reason why the mutant strain became not to form pyocyanine, the following discussions will be brought up.

1. The mutant strain might acquire such ability as to make the condition for pigmentation unsuitable by lowering pH due to the formation of acid[1].

2. The acquisition of such ability as to form an inhibitory substance of pigmentation, which is often recognizable in natural materials[1,8].

3. The change in bacterial character for pigmentation in getting sensitive to the composition of its cultural medium[1].

4. The essential change in bacterial choractor based on the genetic block in the enzyme system of pyocyanine synthesis.

5. The loss or decrease in the capacity of synthesizing the cofactor required for the enzyme action in pyocyanine synthesis.

Pyocyanine Formation by Mutant Strain

In order to test what effects would be produced on pyocyanine formation in various media of different composition, the experiments shown in Table 2 have

Table 2. Effect of concentration of each medium component on pyocyanine formation.

Strains	Components (%)										
	Glycerol				Peptone			Urea			
	6.0	4.0	2.0	1.0	2.0	1.0	0.5	0.4	0.2	0.1	0.05
B_1	###	###	###	###	+	###	###	###	###	###	###
Bd	−	+	+	+	+	+	−	+	+	+	+
Bx	−	−	−	−	−	−	−	−	−	−	−
C_1	−	−	−	−	−	−	−	−	−	−	−
	K_2HPO_4					$MgSO_47H_2O$					
	0.1	0.05	0.025	0.010	0.005	0.1	0.05	0.025	0.010	0.005	
B_1	−	+	###	###	###	###	###	###	###	+	
Bd	−	+	+	+	+	+	+	+	−	−	
Bx	−	−	−	−	−	−	−	−	−	−	
C_1	−	−	−	−	−	−	−	−	−	−	

Basal medium was the same as in Table 1. Experiments were carried out with the media containing requisite amount of one component and other materials of the same concentration as in basal medium. Incubation temperature and period were the same as in Table 1.

been performed. It was found that one of the reasons why pigmentation did not take place was attributable to the formation of acid in the glycerol medium with Strain C_4, while any kinds of the media did not support the formation of pyocyanine with other mutant strains.

If the concept stated in Clause 2 may be reasonable, it should be resulted that when the cultured solution of the mutant strain is added to the medium of the normal strain, pyocyanine formation will not be revealed as in the case of the mutant strains.

However, this was not the case. Pyocyanine formation was rather remarkably promoted by administering the product of the mutant strain. This fact rather supports the idea in Clause 4, suggesting that pyocyanine formation stops midway at some reaction step to accumulate the intermediate which can further be reacted by the normal strain into pyocyanine. Similarly, this concept will also be sustained in the possibility that by the administration of the product of the normal strain cultured in the presence of the inhibitory agent, pyocyanine

formation may be revealed by the mutant strain. The following experiment will endorse a pertinency of the above concept. The cultured solution of the normal strain incubated in the glucose medium at 37° for 48 hours was treated with chloroform to eliminate a trace of pyocyanine formed and to kill the bacteria, allowed to stand for several hours after feing suppliemented with glycerol and other nutrients, and pH was adjusted to 7.4. This solution, followed by aspirating chloroform involved, was inoculated with the pigmentless mutant strain and incubated at 37° for 48 hours. As seen in Table 3, pyocynine formation was successfully revealed.

Similar experiment was carried out with the cultured solution of the normal strain grown on excess of phosphate : the cultured solution incubated for 48 hours was saturated with ammonium sulfate and extracted with butanol. The butanol layer dehydrated was transferred into a small amount of aqueous solution by shaking with excess of ethyl ether and then the solvent involved was evaporated. The experiment with the medium to which this extract was added, was found to be more successful in demonstrating the formation of pyocyanine by the mutant (Table 3). Similar result has also been observed with the case

Table 3. Pyocyanine formation by mutant strain.

Experimental number		No. 1	No. 2	No. 3	Control
Strains	C_1	0.001	0.006	0.003	—
	Bd	0.004	0.014	0.010	0.002
	Bx	—	—	—	—

Basal medium was composed of 2% glycerol, 0.2% urea, 0.05% $MgSO_4 7H_2O$, 0.025% K_2HPO_4 and 0.0005% $Ee_2(SO_4)_3$.

Inculation was carried out at 37° for 48 hours. Pyocyanine was expressed in %. No. 1 expresses the medium containing the accumulation product of the bacteria grown on glucose, Nos. 2 and 3 contain the respective products accumulated in the media of excessive phosphate and of arsenite.

of some other inhibitory agents. These results may not only account for the mechanism of no pigmentation of the mutant strain but also support the possibility that when the inhibitory agent is added to the medium of the normal strain, the intermediate will be accumulated in the medium.

In the above experiment, Strain Bx did not reveal any formation of pyocyanine. This question will be resolved from the interpretation that the accumulation product administered may be situated at the earlier reaction step than the enzyme block in the bacteria.

Pyocyanine Formation in Association System of Mutant Strains

Based on the results shown above, it may be possible to say that no pigmentation phenomenon in mutant strain is ascribable to the enzyme gap in reaction step in pyocyanine synthesis system, and to that genotypes in spontaneous mutant strain may not necessarily be the same with one another. According to these concepts, further experiments were attempted : the culture extract of one

mutant strain was provided to another mutant to be incubated.

Indeed, pyocyanine formation was indicated after the same manner as in the case of the inhibiting process in normal strain.

It is interesting to note that the formation of pyocyanine is permitted only when the systematic assortment is kept between two strains. As seen in Table 4, Strain Bx has never produced pyocyanine from the accumulation product of

Table 4. Pyocyanine formation in association system of mutant strains.

Strains		Acceptors of the accumulation products		
		Bx	C_1	Bd
Donors	Bx	Bx−Bx (−)	Bx−C_1 (++)	Bx−Bd (+++)
	C_1	C_1−Bx (−)	C_1−C_1 (−)	C_1−Bd (++)
	Bd	Bd−Bx (−)	Bd−C_1 (−)	Bd−Bd (+)
Nil		Bx (−)	C_1 (−)	Bd (+)

Basal medium was same as in Table 3.
Incubation period and temperature were the same as in Table 1.

any other strains, suggesting that its blocked step in enzyme system lies beyond that of other strains. In this case, C_1 did not always exhibit pyocyanine formation in proportion to the amount of the product administered by Bx. For the explanation of this phenomenon, it is postulated that Strain C_1 has two defective enzyme steps : the one is perfect enzyme gap which is situated at an earlier step than that of Strain Bx, the other is weakly blocked at the step beyond that of Bx, as can also be considered with Strain Bd which produces a small amount of pyocyanine by itself. Based on these concepts, the scheme expressed in Fig. 2 will be proposed.

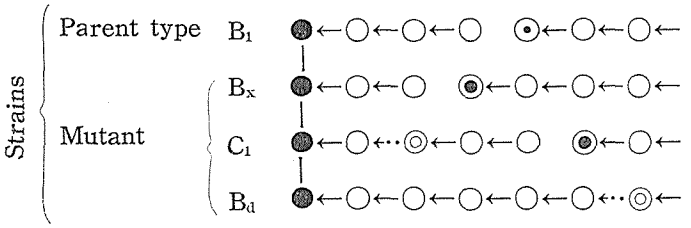

Fig. 2 Enzyme block in pyocyanine synthesis of mutant strain.
● pyocyanine, ⊙ intermediate accumulated in the presence of inhibitor, ◉ do due to a perfect enzyme gap in mutant stsain, ◎do at a weakly blocked step in mutant strain.

On the other hand, differing from the experiment mentioned above, in the mixed culture of two mutant strains according to a syntrophic system pyocyanine was hardly produced, probably on account of the fact that only one strain grew predominantly by overwhelming the other.

In the experimental results described above, another question has been remained concerning the mechanism of spontaneous mutation : why would the genetic block happen at a different step in reaction sequence of the enzyme system, even in the mutation occurred under the same condition? Accordingly, it may

be impossible to make the mutation direct toward a certain desirable phenotypical expression of the bacteria as long as the mutation occurs through a random process. Inconnection with this problem, the following experiment has been designed.

Induction of Mutation

As a physical method for an inducation of bacterial mutation, such radiations as X-rays, gamma rays, neutron or ultraviolet rays have exclusively been adopted.[9-11] The experimental results hitherto mentioned were wholly those that dealed with the strain regarded to be of spontaneous mutation. Even the mutant strain developed through the inducing procedure may be difficult to distinguish from the spontaneous mutation unless it is of prominent feature. The experiment with use of the radiation such as X-rays or ultraviolet rays has not been so successful as had been expected in inducing the mutation of no pigmentation, perhaps owing to the less capacity of the radiation.

For the reason why a mutation occurs spontaneously, it has come to reflect on the injury of heat in daily management of the bacteria. The present bacteria appeared to be killed by heating treatment at 100° for 20 minutes, or at 100° for 5 minutes being accompanied by cooling treatment, and yet occasionally the cell proliferation might be recovered after several days of incubation. From the survivals through this treatment, such a strain was isolated as decreased or lost the ability to form pyocyanine, and simultaneously some strains have come to reveal a colony of metallic luster. As a chemical mutagent,[12] nitrogen mustard, formaldehyde, hydrogen- or organic peroxide and some alkaloids have been known. In the present experiment besides formaldehyde and hydrogen peroxide which were not observed to be effective with the present strain, the following substances were emplyed to test, which have been known in the present study to be an inhibitory agent for pyocyanine formation: 0.02% arsenic acid, 0.01% potassium periodate, 0.05% resorcinol, 0.02% $CoCl_26H_2O$ and 0.01% $CuSO_45H_2O$, which are of limit concentration to permit the bacterial growth in peptone medium. From the agar plate of the bacteria followed by the successive generation culture in the medium containing the above agents, the numerous colonies which reveal no pigmentation have appeared with the exception of the case of $CuSO_4$. This fact could clearly be distinguished from the case of the spontaneous mutation which has generally been known to be about one mutation per 10^6 to 10^8 cells for many of the types of mutation.

However, from the progency of a strain developed through the above treatment, the strain showing the recovery of pigmentation was comparatively frequently derived after the successive cultivation, although it is unknown whether this event is based on the reverse mutation to the parent type or on the fact that this variation is so temporary as to be scarcely inherited.

These phenomena were observed in the case in which streptomycin was used as a mutagenic agent. The strain which has lost the ablity to form pyocyanine by treating with streptomycin exhibited simultaneously the resistance to

it even at its high level, being accompanied by the recovery of the ability for pigmentation. It is of interest to point out that the resistance of the bacteria to streptomycin was also revealed to be induced even in the case of pigmentation. At a lower concentration of streptomycin, the bacteria which had been responsive to its inhibiting action at the same level as above, have become to form pyocyanine in the presence of it, being induced to resist to this agent without suffering the mutagenic effect on the ability to form pyocyanine, differing from the case of high concentration of the agent. In this case, it might be difficult to define whether this variation was a genuinely induced mutation or the selection of the mutation produced spontaneously from the culture of the parent strain.

SUMMARY

1. It has been informed that one strain isolated at the start of the experiment of this series, numerous variant strains were spontaneously derived, of which the majority was regarded as genetic variation, and that these strains might be applicable to the study on the mechanism of biosynthesis of pyocyanine.

2. The possibility was pointed out from the experiment with the mutant strain that when pyocyanine was not permitted to form in the medium of the normal strain cultured in the presence of inhibitory agent, an intermedlate product in pyocyanine synthesis could be accumulated in this medium.

3. The accumulation of the intermediate product might be demonstrated with the mutant strain, from the fact that when the product of the one mutant strain was administered to the other mutant in a systematic assortment of two strains, pyocyanine could be revealed in spite of the loss of their ability for pigmentation.

4. From the requirement of the orderly assortment between two strains, the accumulation products of the mutant strains are considered to be not necessarily identical with each other, suggesting that the gengtic blocks of the enzyme system in pyocyanine synthesis are shown at a different reaction step, similarly to the phenomenon that the accumulation products of the normal strains may be different from one another, according to the kind of inhibitors.

5. It has been shown that through the treatment of the bacteria with a mutagenic agent, inability for pyocyanine formation or resistances of the growth and of the pigmentation to the agent was induced, and discussed whether this phenomenon would be distinguished from the selection of spontaneous mutation, according to the circumstances to be concerned with the induction of the mutation.

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